Vol. 93, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 28, 1980 Pages 403-408

AMINO ACID SEQUENCE AT THE PHOSPHORYLATED SITE OF RAT LIVER PHENYLALANINE HYDROXYLASE AND PHOSPHORYLATION OF A CORRESPONDING SYNTHETIC PEPTIDE

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Received February 1,1980

SUMMARY

Purified rat liver phenylalanine hydroxylase was phosphorylated by incubation with (^{32}P)ATP and the catalytic subunit of pig muscle cyclic AMP-stimulated protein kinase. After digestion of the phosphorylated phenylalanine hydroxylase with pepsin, one major (^{32}P)phosphopeptide with the amino acid sequence Ser-Arg-Lys-Leu-(^{32}P)SerP-Asx-Phe-Gly-Glx-Glx was isolated. Two non-phosphorylated octapeptides, Ser-Arg-Lys-Leu-Ser-Asp-Phe-Gly and Ser-Arg-Lys-Leu-Ser-Asn-Phe-Gly, were synthesized and characterized as substrates of the protein kinase.

INTRODUCTION

Purified rat liver phenylalanine hydroxylase (EC 1.14.16.1), a tetrameric enzyme with a subunit molecular weight of 51,000 (1), is a substrate of cyclic AMP-stimulated protein kinase. The phosphate incorporation is accompanied by an increase of the enzyme activity when assayed in the presence of the natural cofactor tetrahydrobiopterin (2). In addition, subcutaneous or intraperitoneal administration of glucagon to rats results in a similar change of the enzyme activity, indicating that a phosphorylation-dephosphorylation reaction is one method of regulation of this enzyme in vivo (3).

Previous reports have demonstrated the importance of certain features in the primary structure of protein substrates of cyclic AMP-stimulated protein kinase. The phosphate acceptor, usually a seryl residue, is often preceded on the amino terminal side by one or two arginines, separated from the serine by one or two amino acids (4,5,6). Recently Kemp has demonstrated the relative importance of arginines in the first three positions on the amino terminal side of the serine in peptides related to the sequence of rabbit cardiac troponin inhibitory subunit (7). However, not all substrates conform to this model, and we therefore considered it of interest to isolate and characterize the phosphorylatable site of phenylalanine hydroxylase.

0006-291X/80/060403-06$01.00/0

MATERIALS AND METHODS

DEAE-cellulose (DE-52) was purchased from Whatman Ltd., hydroxyl apatite and AG 1-X8 from Bio-Rad Laboratories and Sephadex gels from Pharmacia Fine Chemicals. (^{32}P)ATP was obtained from New England Nuclear, pepsin (type EPBK) from Boehringer Mannheim and Histone II AS from Sigma. All other chemicals were of reagent grade.

Catalytic subunit of cyclic AMP-stimulated protein kinase from pig muscle was prepared essentially according to the method of Bechtel et al. (8,9) from the DEAE-cellulose peak 1 through the CM-Sephadex step. One unit of protein kinase activity was defined as the amount of enzyme catalyzing the incorporation of 1 pmol of (^{32}P)phosphate per minute into mixed histones (7.5 mg/ml) from 0.1 mM (^{32}P)ATP at pH 6.9 and 30°C.

Radioactivity was measured as Cerenkov radiation as described by Mårdh (10).

Phenylalanine hydroxylase activity was assayed as described by Gillam et al. (11) with a final incubation volume of 0.5 ml, and tyrosine was measured according to the method of Udenfriend and Cooper (12). $A^{0.1\%}_{280}$ = 1.0 was used for protein determinations.

Phenylalanine hydroxylase was purified according to the method of Kaufman and Fisher (1) through the first ammonium sulphate precipitation. The precipitate was dissolved in a buffer containing 50 mM Tris HAc, pH 7.0, 10 mM phenylalanine, 1.0 mM EDTA, 0.1 mM dithiothreitol and 5% (v/v) glycerol. After dialysis for 4 hours against 100 volumes of this buffer, the protein was applied to a 3.5 x 15 cm column of DE-52, which was washed with the same buffer and eluted with a linear gradient from 0 to 400 mM of sodium acetate (total volume 500 ml). The fractions showing enzyme activity were pooled and applied to a 2 x 3 cm hydroxyl apatite column equilibrated with 1 mM potassium phosphate, pH 7.0, 300 mM potassium chloride, 10 mM phenylalanine, 1.0 mM EDTA, 0.1 mM dithiothreitol and 5% (v/v) glycerol. Elution was achieved with a linear gradient (total volume 100 ml) from 1 to 150 mM potassium phosphate. The enzyme was by now approximately 50% pure and free from phosphorylatable contaminations, as judged from sodium dodecyl sulphate gel electrophoresis.

50 ml of this phenylalanine hydroxylase (A_{280} = 1.05) were phosphorylated with 1 mM (^{32}P)ATP (specific radioactivity 12,000 cpm/nmol) and 15,000 units of protein kinase. The incubation mixture also contained 1.0 mM EDTA, 0.1 mM dithiothreitol, 300 mM potassium chloride, 5% (v/v) glycerol, 5.0 mM magnesium acetate and 100 mM potassium phosphate pH 7.0. After 30 minutes the incubation was interrupted by gel filtration on a 5.5 x 45 cm column of Sephadex G-50, equilibrated and eluted with 5 mM potassium phosphate, pH 7.0.

To the phosphorylated enzyme, 1 M HCl and pepsin were added to final concentrations of 0.05 M and 0.5 mg/ml, respectively. Digestion took place for 2 hours at 25°C, and was interrupted by chromatography on a 7.0 x 55 cm column of Sephadex G-25, equilibrated and eluted with 50 mM pyridine-acetic acid buffer, pH 3.1. The radioactive material appeared as a symmetrical peak after 0.6 column volume. The pooled radioactive material was further purified on a 0.9 x 30 cm column of SP-Sephadex C-25, equilibrated with the same buffer as above and eluted with a linear gradient from 50 to 300 mM (200+200 ml) pyridine-acetic acid buffer, pH 3.1. The radioactive peak fractions were pooled, lyophilized and redissolved in 25 mM ammonium carbonate buffer, pH 8.5. The final step in the purification procedure was chromatography on a 0.9 x 30 cm QAE-Sephadex A-25 column equilibrated with 25 mM ammonium carbonate buffer, pH 8.5, and eluted with a linear

gradient from 25 to 500 mM ammonium carbonate buffer, pH 8.5, in a total volume of 400 ml.

Purified (^{32}P)phosphopeptide was hydrolyzed for 24 hours at 110°C in sealed ampoules containing 6 M HCl and 1% phenol. The amino acid composition was determined using a single-column Durrum amino acid analyser (Durrum D-500). The values for serine and threonine were corrected for losses during hydrolysis.

Amino acid sequence analysis was carried out according to the method of Hartley (13). The radioactive ^{32}P-label was located by high voltage electrophoresis followed by autoradiography as described in (9).

Two corresponding nonphosphorylated octapeptides were synthesized by the solid phase method as described by Zetterqvist et al. (4). Asparagine was incorporated as the corresponding xanthydryl derivative with dicyclohexylcarbodiimide. The purity of the peptides was verified by amino acid analysis.

Without further purification, the synthetic peptides were phosphorylated in an incubation mixture containing 0.1 mM (^{32}P)ATP, 1.0 mM magnesium acetate, 5 mM potassium phosphate buffer, pH 7.0 and 2 units of protein kinase. The incubation was carried out for 5 minutes at 30°C in a final volume of 50 µl, and was interrupted by the addition of 25 µl of glacial acetic acid. Excess (^{32}P)ATP and (^{32}P)orthophosphate were removed by chromatography on 0.4 x 5 cm columns of AG 1 - X8, equilibrated and eluted with 30% acetic acid as described by Kemp et al. (14). The resulting Lineweaver-Burk plots were used to calculate apparent K_m and V_{max} values.

The phosphorylation of phenylalanine hydroxylase was studied in the presence of 80 mM potassium phosphate, pH 7.0, 1.0 mM magnesium acetate, 400 units of protein kinase and 0.1 mM (^{32}P)ATP in a volume of 250 µl. The incubation continued for 5 minutes at 30°C and was interrupted by the addition of 2 ml of ice cold 10% (w/v) trichloroacetic acid. 1 mg of bovine serum albumin was added and the protein was collected by centrifugation for 5 minutes and redissolved in 500 µl of 0.2 M sodium hydroxide. The protein was again precipitated with 10% (w/v) trichloroacetic acid, centrifuged and redissolved in sodium hydroxide. This process was repeated twice, after which the radioactivity was measured and the phosphate incorporation calculated.

RESULTS AND DISCUSSION

The yield of (^{32}P)labelled phosphoprotein from the phosphorylation of phenylalanine hydroxylase was 163 nmoles. 37% of this was recovered as one major phosphopeptide after the last step in the purification procedure (Fig. 1). The amino acid analysis of this peptide (Table I) showed a peptide content at least twice that calculated from the radioactivity of the sample, indicating a substantial amount of endogenous phosphate in the phenylalanine hydroxylase, which is in accordance with results previously published by Abita et al. (2). A minor radioactive peak appeared early in the chromatogram. It was shown by amino acid analysis to be impure and was not further studied.

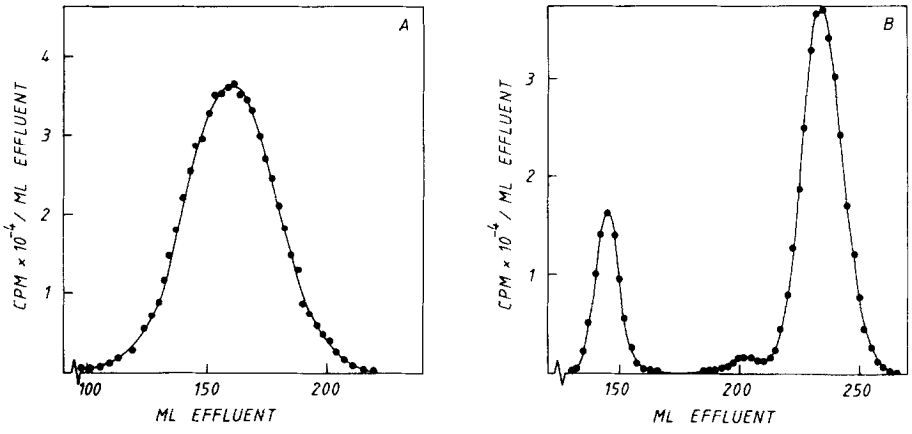

Fig. 1. Two successive chromatographies of ^{32}P-labelled peptide material from rat liver phenylalanine hydroxylase digested with pepsin. The numbers on the abscissa refer to the effluent collected from the start of the gradient. A represents the SP-Sephadex G-25 step and B the QAE-Sephadex A-25 step. The conditions of both chromatographies are described in Materials and Methods.

The result of the amino acid sequence analysis was the peptide Ser-Arg-Lys-Leu-Ser-Asx-Phe-Gly-Glx-Glx. High voltage electrophoresis revealed that the (^{32}P)phosphate left the peptide in the same step as the second serine was cleaved off, thus identifying this amino acid residue as the phosphorylated one.

Two corresponding non-phosphorylated peptides were synthesized:
A. Ser-Arg-Lys-Leu-Ser-Asn-Phe-Gly and B. Ser-Arg-Lys-Leu-Ser-Asp-Phe-Gly.

TABLE I

Amino acid composition of the major phosphopeptide

Amino acid	Nmol analysed	Nmol analysed / 21.0	Nearest integer
Aspartic acid	23.71	1.13	1
Threonine	1.75	0.08	
Serine	37.28	1.78	2
Glutamic acid	44.05	2.10	2
Proline	-	-	
Glycine	21.06	1.00	1
Alanine	2.11	0.10	
Valine	1.56	0.07	
Methionine	-	-	
Isoleucine	0.99	0.04	
Leucine	21.12	1.01	1
Tyrosine	1.21	0.06	
Phenylalanine	19.56	0.93	1
Histidine	1.48	0.07	
Lysine	21.25	1.01	1
Arginine	18.38	0.88	1

Table II

A comparison between the rate of phosphorylation of the peptides and phenylalanine hydroxylase. The incubation mixture contained 0,1 mM (^{32}P)ATP, 1 mM magnesium acetate and 20 mM potassium phosphate, pH 7,0. The incubation volume was 80 µl. Due to the great differences in the rate of phosphorylation between the substrates, different amounts of protein kinase were used. The phosphorylation of histone (4 mg/ml) was used as reference.

Substrate	Substrate concentration µM	Amounts of protein kinase used units	Rate of (^{32}P) incorporation pmol/min	Relative rate of phosphorylation %
Histone		190	123	100
Phenylalanine hydroxylase	2	1900	14	1
Peptide A	80	19	66	550
Peptide B	80	19	44	350

The peptide contents of the lyophilized preparations were 68 and 61% respectively, and the relative amounts of amino acid residues were: Ser = 2.0, Arg = 1.0, Lys = 1.0, Leu = 0.9, Asn or Asp respectively = 1.0, Phe = 1.1, Gly = 1.0. No attempts were made at this stage to prepare smaller substrates since such experiments have been made earlier in connection with studies on pyruvate kinase (4).

Studies of the phosphorylation of phenylalanine hydroxylase and the two synthetic peptides showed, that whereas the apparent K_m for phenylalanine hydroxylase was approximately 2 µM, the apparent K_m values for the A- and the B-peptide were in the order of 0.1 and 0.3 mM respectively. The precise values for the peptides were somewhat difficult to determine, since a pronounced substrate inhibition was detected at a substrate concentration as low as 100 µM. The rate of phosphorylation of the substrates was therefore compared, using concentrations near the K_m values of the A-peptide and the phenylalanine hydroxylase, i.e. 80 µM for the peptides and 2 µM for the enzyme. The result is shown in Table II.

CONCLUSIONS

The amino acid sequence at the phosphorylatable site of phenylalanine hydroxylase contains two basic residues separated from the phosphorylatable serine by one amino acid. This sequence exhibits an amino acid pattern similar to that of the phosphorylatable site of pyruvate kinase and the α-subunit of phosphorylase kinase, but differs in having Arg-Lys-

X-SerP instead of Arg-Arg-X-SerP. Two synthetic octapeptides with related sequences were phosphorylated with the apparent K_m-values of 0,1 and 0,3 mM, the latter value referring to the peptide with a negative charge on the amino acid after the serine. These values are about two orders of magnitude higher than the apparent K_m for the phenylalanine hydroxylase, indicating that other structures in the phenylalanine hydroxylase might be of importance for recognition by the protein kinase as well as the primary sequence just around the phosphate accepting serine.

ACKNOWLEDGEMENTS

This investigation was supported by the Swedish Medical Research Council (Project No. 13X-50) and the Swedish Natural Science Research Council (Project No. K 3020-101). The amino acid analyses were kindly performed by Dr. D. Eaker and Mr. R. Thorzelius at the Institute of Biochemistry, Uppsala. The skilled technical assistance of Mrs. Imma Brogren is gratefully acknowledged.

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